Remarks

Claims 49-53 and 56-79 are pending. Claims 51, 54 and 55 have been newly cancelled. Claims 49-53 and 56-57 have been newly amended. Claims 58-79 are newly added. Support for these amendments are found throughout the specification and in the claims as originally filed. No new matter has been entered. All newly added claims are encompassed by Group I of the restriction requirement drawn to methods of identifying biomarkers and methods for diagnosis and prognosis of bladder cancer, further restricted to the TNFRSF7 gene.

Claims 71, 72, 73, 74 and 77 clarify that said levels of RNA encoded by said gene are in blood samples leukocytes which include all of the types of leukocytes in whole blood, i.e. of blood samples which include granulocytes in addition to mononuclear cells (T-lymphocytes, B-lymphocytes and monocytes). This phrase finds clear support in the specification, including at Figure 5C which shows standardized levels of insulin gene in each of the fractions of leukocytes which collectively constitute unfractionated leukocytes, i.e. granulocytes, T-lymphocytes, B-lymphocytes and monocytes (labeled "G.R.", "CD 3+", "CD19" and "MONO", i.e., respectively). It is well known to the ordinarily skilled artisan that CD3 and CD19 are specific cell surface markers of T-lymphocytes and B-lymphocytes (refer, for example, to the enclosed Abstract of Casey *et al.*, 1988. simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human hematopoietic and lymphoid tissues. Am J Pathol. 131:183-9). The fact that granulocytes (G.R.), lymphocytes [T-lymphocytes (CD 3+) and B-lymphocytes (CD19+)] and monocytes (MONO) represent all of the types of leukocytes found in blood is taught at Fig. A.23 Immunobiology. Garland Publishing. 2001. Fifth Edition. Janeway, Travers, Walport, and Shlomchik, eds. (attached) which clearly teaches that leukocytes are composed of granulocytes and mononuclear cells, and that the latter are composed of lymphocytes and monocytes. Additional support for the term "leukocytes" is found at paragraphs [0003] and [0086] of US20040241726, the Published Application.

New independent claim 79 claims a method of classifying gene expression in a test subject relative to a population of control subjects that includes subjects having bladder cancer and healthy subjects. New claim 79 comprises a step of quantifying a level of RNA encoded by a TNFRSF7 gene in a blood sample from the test subject, and a subsequent step of comparing the

level in the sample from the test subject with levels of RNA encoded by the gene in blood samples from the subjects having bladder cancer and in blood samples from the healthy subjects. The new claim concludes that a determination that the level in the sample from the test subject is statistically similar to the levels in the samples from the subjects having bladder cancer and is statistically different from the levels in the samples from the healthy subjects classifies the level in the sample from the test subject with the levels from the samples from the subjects having bladder cancer; and/or concludes that a determination that the level in the sample from the test subject is statistically different from the levels in the samples from the subjects having bladder cancer and is statistically similar to the levels in the samples from the healthy subjects classifies the level in the sample from the test subject with the levels in the samples from the healthy subjects. Support for reciting comparison of biomarker RNA levels of a test subject with those of control subjects having a disease (i.e. bladder cancer) and with those of healthy control subjects, and determination of a statistically significant similarity or difference therebetween can be found in the published application US *20040241728* (hereinafter "Published Application"), for example at paragraph [0125] ("*when comparing two or more samples for differences, results are reported as statistically significant when there is only a small probability that similar results would have been observed if the tested hypothesis (i.e., the genes are not expressed at different levels) were true*"), at paragraph [0126] ("*when comparing two or more samples for differences, results are reported as statistically significant when there is only a small probability that similar results would have been observed if the tested hypothesis (i.e., the genes are not expressed at different levels) were true*"). Support for reciting classification of a test subject level with specific control levels can be found, for example, at claim 12 as originally filed ("*d) determining whether the level of said one or more gene transcripts of step a) classify with the levels of said transcripts in step b) as compared with the levels of said transcripts in step c)*"), at paragraph [0133] (relating to "*Methods that can be used for class prediction analysis*"), [0351] ("*Blood samples were taken from patients who were diagnosed with bladder cancer as defined herein. Gene expression profiles were then analyzed and compared to profiles from patients unaffected by any disease.*").

Claims Rejection - 35 U.S.C. 112 2nd

Claims 51-57 are rejected under 35 U.S.C. 112, 2nd paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

The office action indicates that the recitation of "unfractionated samples of lysed blood" is indefinite. Although Applicant respectfully traverses, Applicant has canceled independent claim 51 and dependent claims 54 and 55 solely for the purposes of advancing prosecution without prejudice for pursuing the unclaimed subject material in another application, rendering the rejection of claims 51, 54 and 55 moot. Applicant has amended dependent claims 52-53 and 56-57 to be dependent from claim 49 or newly added claim 60, which do not recite the phrase "unfractionated samples of lysed blood".

Claims Rejection - 35 U.S.C. 112 1st

Claims 51-57 are rejected under 35 U.S.C. 112, 1st paragraph, as failing to comply with the written description requirement on the grounds that the instantly recited phrase "unfractionated samples of lysed blood" is new matter. Although Applicant respectfully traverses, Applicant has canceled claim 51 and dependent claims 54 and 55 solely for the purposes of advancing prosecution without prejudice for pursuing the unclaimed subject material in another application, rendering the rejection of claims 51, 54 and 55 moot. Applicant has amended dependent claims 52-53 and 56-57 to be dependent from claim 49 or newly added claim 60, which do not recite the phrase "unfractionated samples of lysed blood".

Claims 49-57 are rejected under 35 U.S.C. 112, 1st paragraph, as failing to comply with the enablement requirement.

Applicant respectfully traverses. Applicant disagrees with the rejection's assertion that the skilled artisan would have required an undue amount of experimentation to make and/or use the claimed invention in view of the breadth of the claims and the lack of guidance provided by the specification as well as the unpredictability of the art.

The rejected claims include the steps of determining the level of RNA encoded by a TNFRSF7 gene in a blood sample obtained from a human test subject and comparing it to the

level of control RNA encoded by the TNFRSF7 gene in blood samples from control subjects, wherein the comparison is indicative of bladder cancer in said human test subject.

Applicant specifically traverses the statement on page 4 of the office action that "the independent claim, as written, states that a comparison of a human test subject TNFRSF7 RNA level in a blood sample to a control indicates that bladder cancer is present in the test subject", and the statement on page 5 of the office action that the "claims are extremely broad because they set forth that any or all comparison between a test subject and RNA level from "control subjects" is indicative of disease". Applicant clarifies that the phrase "wherein said comparison of said quantified level of step (a) with said quantified level of said control subjects is indicative of bladder cancer in said human test subject" of independent claim 49, is a narrowing limitation, limiting the claim to only those comparisons which are indicative of the test subject having bladder cancer, and excluding those comparisons which do not indicate that the test individual has bladder cancer.

However, in the interest of expediting prosecution, Applicant has added new claims which more clearly reflect the intention of the newly cancelled claims. Specific points raised in the instant enablement rejection will be addressed to the extent they are relevant to the newly added claims.

The rejection asserts that the claims are broad with respect to "control subjects", indicating that "control subjects" could encompass patients with bladder cancer, healthy patients, and patients with some other disease such as lymphoma or a particular stage of bladder cancer (page 5 of the office action). The instant claims recite two clearly defined sets of controls; patients having bladder cancer and healthy controls. At least one claim, claim 63, limits the controls to healthy subjects.

The rejection asserts that the claims are very broad in scope because they encompass that any level and direction of difference in gene expression between the tested subjects is indicative of disease, (page 5 of the office action). As described above, Applicant disagrees with this claim interpretation. Accordingly, Applicant has newly added claims which specify a direction and a level of difference in TNFRSF7 expression required to be detected between the blood samples of the test subject and the healthy controls. For example, claim 75 recites "wherein said test subject is a candidate for having if the level of RNA encoded by said TNFRSF7 gene in said blood

sample of said human test subject is at least 3 times *lower* than that of said healthy subjects with a p value less than 0.05", (emphasis added). Such a statistical probability will not likely be achieved comparing one test subject with only two control subjects, thereby addressing the concern raised in the instant rejection over the minimum number of controls necessary for a meaningful comparison at pages 6 and 9 of the instant office action.

By reciting that the controls are healthy subjects, newly added claim 75 also addresses the issue raised in the instant rejection concerning detecting bladder cancer in a test subject based on a comparison between the test individual and control individuals where the control individuals don't have bladder cancer, but could still have some other disease or condition, as suggested at page 5 of the office action. Applicant respectfully traverses the assertion on the last paragraph of page 7 of the office action that "there is no guidance or analysis of data in the specification to suggest that this gene in particular is sufficient to conclude that bladder cancer is present". Applicant submits that this gene is indeed sufficient to provide an indication of bladder cancer on the grounds that the specification discloses that RNA encoded by the TNFRSF7 gene in a blood sample of from a bladder cancer patient is differently expressed relative to healthy subjects with a p value = 0.04, see Example 19, Figure 17 in which five patients with bladder cancer and 18 control individuals not having bladder cancer were analyzed. In addition, Applicant's position is clearly supported by the attached declaration filed under 1.132 which discloses post-filing validation experiments using both quantitative RT-PCR (QRT-PCR), an alternate technology relative to microarray analysis employed in the experiments disclosed at Example 19 of the specification, and using an independent cohort of control and disease subjects relative to those employed in the experiments disclosed at Example 19 of the specification, as well as relative to those employed in Osman *et al.*, of record. The experiments disclosed in the declaration clearly show that RNA encoded by the gene TNFRSF7 is present at statistically lower levels in blood of subjects having bladder cancer relative to healthy control subjects.

The rejection also asserts that the specification does not establish any particular level of expression of TNFRSF7 which is sufficient to detect bladder cancer to the exclusion of other disorders, at page 8 of the office action.

While not necessarily agreeing that the claims categorically exclude all other disorders, solely for the purpose of expediting prosecution, Applicant has included the limitation in claim

75 that the recited comparison between a test subject and controls indicates that the test individual is a "candidate" for having bladder cancer.

The office action specifically contends that the teachings of Showe *et al.* render unpredictable whether a different expression level of TNFRSF7 relative to healthy patients indicates bladder cancer or cutaneous T-cell lymphoma (CTLC).

Applicant wishes to point out that paragraph [0106] of Showe et al.states that all of the genes set forth in Table 1, i.e. including TNFRSF7, of this reference are upregulated in disease relative to controls. As is taught in Osman *et al.*, of record (page 3378, last paragraph), and in the attached declaration under 1.132, however, expression of TNFRSF7 is downregulated in blood of bladder cancer patients relative to controls. Additionally, Showe et al. refers to experimental data purportedly demonstrating that TNFRSF7 is differently expressed in purified PBMCs, i.e. in an isolated fraction of leukocytes, but does not refer to any experimental data showing that TNFRSF7 is differently regulated in whole blood, i.e. in blood which is not fractionated into cell types or in leukocytes which are not fractionated into cell types, as is taught in Example 19 of the specification, and as is instantly claimed. In addition, Applicant has amended claim 49 such that similarity of TNFRSF7 gene expression in a test subject to that in bladder cancer patient controls, as opposed to healthy controls, is indicative of bladder cancer in the test subject. Such an expression pattern can not be taken as a possible indication of CTLC since the expression of TNFRSF7 in blood of bladder cancer patients is downregulated, as opposed to being upregulated in CTLC patients, relative to healthy controls. In addition, Applicant has newly added claim 79 which recites that expression of TNFRSF7 in blood of a test subject is classified with that of bladder cancer patient controls if it is similar to that in such controls and different relative to that in healthy controls, and *vice versa*, thereby addressing the rejection's concerns with respect to CTLC.

The office action indicates that it would take undue experimentation to practice the invention, specifically to determine difference thresholds required to determine that a patient has or does not have disease, pages 9 and 11 of the office action, and that the invention is in an area that is highly unpredictable, page 11 and throughout the office action. Applicant respectfully disagrees. MPEP 2164.03 indicates that "the "predictability or lack thereof" in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed

invention. If one skilled in the art can readily anticipate the effect of a change within the subject matter to which the claimed invention pertains, then there is predictability in the art." Because of the guidance in the specification which shows a statistically significant correlation between the levels of TNFRSF7 RNA in blood of diseased vs. healthy controls, Applicant contends that one of skill can reasonably predict with a statistically significant probability that a patient may be a candidate for having bladder cancer based on the teachings in the specification.

The office action states that Lee teaches that data obtained from microarrays must be replicated in order to screen out false positive results; that Cheung et al. (2003) teaches that there is natural variation in gene expression amongst different individuals; that Wu et al (2001) teaches that gene expression data, such as microarray data, must be interpreted in the context of other biological knowledge, and that the conclusions that can be drawn from a given set of data depend on the particular choice of data analysis; and Newton et al. (2001) teaches that a replication of data is required for validation.

The office action further contends that the claims may not be enabled on the grounds that post-filing reference Osman *et al.* teaches that TNFRSF7 was found to be differently regulated in bladder cancer patients using only one cohort, and on the grounds that this reference does not explicitly teach that this gene can be used to distinguish between bladder cancer patients and healthy controls.

While disagreeing with the office action's contention based on Newton *et al.* that data replication would be necessary to enable the claims, the attached declaration filed under 1.132 discloses post-filing validation experiments using both quantitative RT-PCR (QRT-PCR), an alternate technology relative to microarray analysis employed in the experiments disclosed at Example 19 of the specification, and using an independent cohort of control and disease subjects relative to those employed in the experiments disclosed at Example 19 of the specification, as well as those employed in Osman *et al.*, of record. The experiments disclosed in the declaration clearly show that RNA encoded by the gene TNFRSF7 is present at statistically lower levels in blood of subjects having bladder cancer relative to healthy control subjects. The experimental data disclosed in the declaration clearly addresses the office action's concerns whereby the claims may not be enabled in view of Osman *et al.* teaching that the results disclosed therein are intrinsic to the particular cohort used in this reference. With regard to Osman *et al.* Applicant

wishes to point out that this reference clearly concludes, despite any conservative academic reservations expressed therein, that biomarkers such as TNFRSF7 can be used to differentiate bladder cancer patients from those having other genitourinary cancers or healthy controls. Namely, this reference clearly teaches that on balance the biomarkers disclosed in this reference are "novel blood biomarkers of human urinary bladder cancer" (title) and can "distinguish bladder cancer from other types of genitourinary cancer and healthy controls" (abstract). Applicant additionally wishes to point out that the mere fact that the scope of the teachings of Osman *et al.* is directed towards biomarker combinations so as to not explicitly conclude that TNFRSF7 alone is sufficient to distinguish bladder cancer from healthy controls, or other genitourinary diseases, does not render the claims non-enabled, which are based on the distinct teachings of the specification.

Applicant respectfully disagrees with the contention in Wu et al. that expression data needs to be interpreted in view of other biological knowledge. Differential gene expression which is reproducible, and is correlated with the state of health or disease of the individual does not necessarily result directly from the state of disease of the individual. Rather these changes in expression can be as a result of a downstream effect of pathogenic processes, and it is not necessary that the biological relevance of the data be known to allow this difference in expression to be useful as a biomarker. For example prostate-specific phosphatase and prostate-specific antigen (PSA) were long used as biomarkers without an understanding of their function (refer, for example, to the enclosed abstracts of: Chu TM, 1990, Prostate cancer-associated markers. Immunol. Ser. 53:339-56; and Diamandis EP., 2000, Prostate-specific antigen: a cancer fighter and a valuable messenger? Clin Chem. 46:896-900).

The Examiner also argues, on the basis of post-filing art of Wu (2001) and Newton (2001), that many factors may influence the outcome of the data analysis and notes that conclusions depend on the methods of data analysis. While considerations such as variability, and normalization are of importance, these considerations are well understood by a person skilled in the art and have been applied for many years to permit development of biomarkers which are indicative of disease. These challenges are well understood, as are the routine experiments required to exemplify statistically significant differences in populations.

Applicant notes that the results disclosed by Cheung *et al.* cannot be reliably extrapolated to primary blood samples since the lymphoblastoid cells employed by Cheung *et al.* are significantly modified relative to primary blood cells, due to being cultured cell lines generated by immortalization of primary human cells derived from "CEPH" families, as indicated in Reference no. 10 of Cheung *et al.* (Dausset *et al.*, 1990. Genomics 6:575; enclosed) at p. 575, right column, 1st paragraph. Applicant notes that immortalized cultured cell lines such as the lymphoblastoid cells taught by Cheung *et al.* undergo significant genetic modification such as strong genome-wide demethylation (refer, for example, to enclosed abstract of: Vilain *et al.*, 2003. DNA methylation and chromosome instability in lymphoblastoid cell lines. Cytogenet Cell Genet. 90:93), as a result of extensive *in-vitro* culturing in the absence of immune or apoptotic mechanisms which function to eliminate mutated cells in the body. As such, immortalized CEPH lymphoblastoid cells may represent a particularly unsuitable cell type for modeling gene expression variability in primary blood cells.

To the extent that Cheung et al. could still be considered to suggest that larger populations of diseased and control populations may be useful to determine what level of differential expression is indicative of disease amongst the population at large, the Applicant submits that the extension of the experiments as outlined in the specification to additional individuals is merely routine. As is noted in Re Wands "*even a considerable amount of experimentation is permissible to practice the claimed methods, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.*" (Re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)).

Furthermore, the decision *In re Angstadt*, 190 U.S.P.Q. 218 (C.C.P.A. 1976) clearly states that even in an unpredictable art, and clearly permits the presence of a screening step to identify those embodiments which possess the desired activity is permissible. In fact, in *Angstadt*, the Court specifically dismissed the notion that the specification must provide a level of guidance that would predict the outcome of an experiment "with reasonable certainty before performing the reaction" and that "such a proposition is contrary to the basic policy of the Patent Act, which is to encourage disclosure of inventions and thereby to promote progress in the useful arts." The "predictability or lack thereof" in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention.

Applicant wishes to point out that in *In re Wands*, the court stated that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. 'The key word is 'undue' not 'experimentation' (citing *In re Angstadt*, 537 F. 2d 498 at 504, 190 U.S.P.Q. 214 at 219 (C.C.P.A. 1976)). The Court also stated that "the test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." (citing In re Jackson, 217 U.S.P.Q. 804 at 807 (Bd. App. 1982)).

As such the Applicants believe there is sufficient guidance provided by the specification and that the art is sufficiently predictable such that the amount of experimentation to perform the subject matter within the instant claims is not undue.

In light of the amendments and above remarks, the Applicant contends that the claims are fully enabled, and respectfully requests reconsideration and withdrawal of the instant rejections.

Conclusion

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. No new matter is added. If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

Date: November 13, 2007

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Encl:

Abstract of: Chu TM, 1990, Prostate cancer-associated markers. Immunol. Ser. 53:339-56; and

Abstract of: Diamandis EP., 2000, Prostate-specific antigen: a cancer fighter and a valuable messenger? Clin Chem. 46:896-900).

Chu TM., 1990. Prostate cancer-associated markers. Immunol Ser. 53:339-56.

Immunodiagnosis of prostate cancer is at a more advanced stage than that of most other tumors. Two well-known markers, prostatic acid phosphatase and prostate-specific antigen, have been used in the clinical management of patients. Prostate-specific antigen is a more sensitive and reliable marker than prostatic acid phosphatase. Serum prostate-specific antigen is effective in monitoring disease status, predicting recurrence, and detecting residual disease. Prostate-specific antigen is a tool for the histological differential diagnosis of metastatic carcinomas, especially in the identification of metastatic prostate tumor cells in distant organs and in the differentiation of primary prostate carcinoma from poorly differentiated transitional cell carcinoma of the bladder. Few data on biological function are available. Prostatic acid phosphatase functions as a phosphotyrosyl-protein phosphatase and prostate-specific antigen as a protease. Physiological function in the prostate remains to be elucidated. Several of the prostate-specific and prostate-tumor-associated antigens, as well as a putative prostate tumor-specific antigen, as recognized by monoclonal antibodies are available. Clinical evaluation of these potential markers is not yet available.

PMID: 1713065 [PubMed - indexed for MEDLINE]

Diamandis EP., 2000. Prostate-specific antigen: a cancer fighter and a valuable messenger? Clin Chem. 46:896-900.

BACKGROUND: Prostate-specific antigen (PSA) is a valuable prostatic cancer biomarker that is now widely used for population screening, diagnosis, and monitoring of patients with prostate cancer. Despite the voluminous literature on this biomarker, relatively few reports have addressed the issue of its physiological function and its connection to the pathogenesis and progression of prostate and other cancers. APPROACH: I here review literature dealing with PSA physiology and pathobiology and discuss reports that either suggest that PSA is a beneficial molecule with tumor suppressor activity or that PSA has deleterious effects in prostate, breast, and possibly other cancers. CONTENT: The present scientific literature on PSA physiology and pathobiology is confusing. A group of reports have suggested that PSA may act as a tumor suppressor, a negative regulator of cell growth, and an apoptotic molecule, whereas others suggest that PSA may, through its chymotrypsin-like activity, promote tumor progression and metastasis. SUMMARY: The physiological function of PSA is still not well understood. Because PSA is just one member of the human kallikrein gene family, it is possible that its biological functions are related to the activity of other related kallikreins. Only when the physiological functions of PSA and other kallikreins are elucidated will we be able to explain the currently apparently conflicting experimental data.

PMID: 10894830 [PubMed - indexed for MEDLINE]

Vilain A, Bernardino J, Gerbault-Seureau M, Vogt N, Niveleau A, Lefrancois D, Malfoy B, Dutrillaux B., 2000. DNA methylation and chromosome instability in lymphoblastoid cell lines. Cytogenet Cell Genet. 90:93-101.

In order to gain more insight into the relationships between DNA methylation and genome stability, chromosomal and molecular evolutions of four Epstein-Barr virus-transformed human lymphoblastoid cell lines were followed in culture for more than 2 yr. The four cell lines underwent early, strong overall demethylation of the genome. The classical satellite-rich, heterochromatic,juxtacentromeric regions of chromosomes 1, 9, and 16 and the distal part of the long arm of the Y chromosome displayed specific behavior with time in culture. In two cell lines, they underwent a strong demethylation, involving successively chromosomes Y, 9, 16, and 1, whereas in the two other cell lines, they remained heavily methylated. For classical satellite 2-rich heterochromatic regions of chromosomes 1 and 16, a direct relationship could be established between their demethylation, their undercondensation at metaphase, and their involvement in non-clonal rearrangements. Unstable sites distributed along the whole chromosomes were found only when the heterochromatic regions of chromosomes 1 and 16 were unstable. The classical satellite 3-rich heterochromatic region of chromosomes 9 and Y, despite their strong demethylation, remained condensed and stable. Genome demethylation and chromosome instability could not be related to variations in mRNA amounts of the DNA methyltransferases DNMT1, DNMT3A, and DNMT3B and DNA demethylase. These data suggest that the influence of DNA demethylation on chromosome stability is modulated by a sequence-specific chromatin structure. Copyright 2000 S. Karger AG, Basel.

American Journal of Pathology, Vol 131, 183-189, Copyright © 1988 by American Society for Investigative Pathology

REGULAR ARTICLES

A simplified plastic embedding and immunohistologic technique for immunophenotypic analysis of human hematopoietic and lymphoid tissues

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Routine fixation and paraffin embedding destroys many hematopoietic and lymphoid differentiation antigens detected by flow cytometry or frozen section immunohistochemistry. On the other hand, morphologic evaluation is difficult in flow cytometric or frozen section studies. A simplified three-step plastic embedding system using acetone-fixed tissues embedded in glycol-methacrylate (GMA) resin has been found to provide both excellent morphologic and antigenic preservation. With our system, a wide variety of antigens are detected in plastic sections without trypsinization or prolonged embedding procedures; pan-B (CD19, CD22), pan-T (CD7, CD5, CD3, CD2), T-subset (CD4, CD8, CD1, CD25) markers as well as surface immunoglobulin and markers for myeloid and mononuclear- phagocyte cells are preserved. In summary, modifications of plastic embedding techniques used in this study simplify the procedure, apparently achieve excellent antigenic preservation, and facilitate evaluation of morphologic details in relation to immunocytochemical markers.

Appendix E

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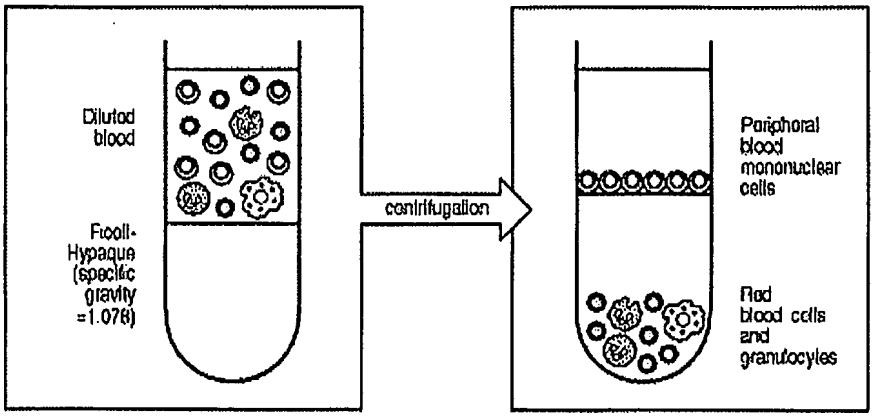

Figure A.23. Peripheral blood mononuclear cells can be isolated from whole blood by Ficoll-Hypaque™ centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque™ and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge through the Ficoll-Hypaque™, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).

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